CORRECTED VERSION

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(54) Title: E4ORF4 AND PP2A POLYPEPTIDES, MODULATORS, AND MIMETICS FOR SELECTIVELY INDUCING CELL DEATH
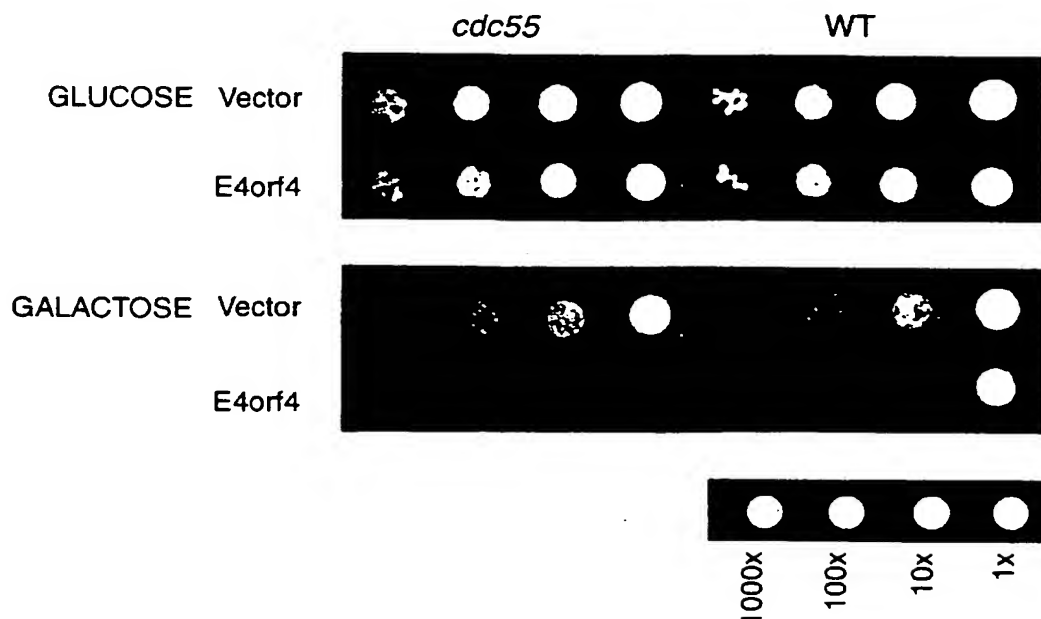
(57) Abstract: Disclosed is the ability of the adenovirus protein, E4orf4, to induce cell death in neoplastic cells, but not in non-neoplastic cells. This discovery allows for the generation of anti-neoplastic therapeutic reagents and compounds. In addition, the invention provides methods for screening for E4orf4 analogs having anti-neoplastic properties.

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E4ORF4 AND PP2A POLYPEPTIDES, MODULATORS, AND MIMETICS FOR SELECTIVELY INDUCING CELL DEATH

5   Background of the Invention

The invention relates to methods for identifying pharmaceutical agents for selectively inducing cell death. Such agents are useful as therapeutics for treating conditions that involve inappropriate cell survival, such as cancer.

Adenoviruses normally infect differentiated epithelial cells of the upper
10   respiratory tract in humans and, in order to replicate, force non-dividing cells into the cell cycle. In humans, the virus eventually kills the host cell.

Rodent cells are non-permissive for viral replication and can become transformed. During adenovirus transformation, the viral E1A proteins promote cellular proliferation and DNA synthesis by binding and inactivating
15   cellular growth suppressors from the Rb and p300 families. In the process of this inappropriate growth stimulation, however, E1A induces the cell to undergo apoptosis. This induction of apoptosis occurs via the activation of the cellular tumor suppressor, p53.

In a normal infection in which apoptosis of the host cell is
20   evolutionarily undesirable, this apoptosis of transformed cells is blocked by the actions of the two viral E1B proteins; the E1B-55K protein binds and inactivates p53, while E1B-19K functions downstream in a manner similar to the cellular Bcl-2 protein.

A second, p53-independent apoptotic pathway induced by E1A is
25   mediated by the E4orf4 protein (see PCT Application No. IB97/01041, published January 15, 1998, hereby incorporated by reference. The involvement of E4orf4 in apoptosis was indicated by our observation that

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adenoviruses lacking E1B-19K could induce apoptosis in cell lines lacking p53. Using viral deletion mutants, we showed that this p53-independent apoptosis relied on expression of another early viral gene product, which was subsequently mapped to the E4 open reading frame 4 protein (E4orf4). This

5   apoptosis could be delayed by expressing either E1B-19K or cellular Bcl-2 protein, but not by E1B-55K. This novel death pathway was found to be activated by adenovirus in both rodent and human cell lines, regardless of p53 expression.

E4orf4 has been shown to interact with PP2A, an abundant

10   serine-threonine phosphatase with pleiotropic activities. The holoenzyme exists in multiple forms as a heterotrimer composed of a catalytic C subunit and A and B regulatory subunits. In mammalian cells, the A and C subunits, which make up the core enzyme, are ubiquitous, each existing in two isoforms. The 65 kDa A subunit is a hook-shaped protein consisting of 15 imperfect

15   repeats of 39 amino acids termed HEAT (huntingtin-elongation-A subunit-TOR) motifs, which are believed to mediate protein-protein interactions. The 37 kDa C subunit is able to bind to repeats 11-15, while the B subunit binds to repeats 1-10.

To date, about 20 B subunits have been cloned; these have been

20   grouped into three classes designated as B, B' and B". The B class proteins are all approximately 55kDa and include at least four members, Bα, Bβ, Bγ and Bδ. Five genes (α, β, γ, δ, ε) producing at least 13 splice variants make up the B' (B56) class, while the B" class contains two alternatively spliced forms of 72 kDa and 130 kDa.

25   The A and C subunits possess phosphatase activity but appear to lack substrate specificity. Specificity appears to be conferred through the various B subunits. Associated B subunits can either inhibit or promote phosphatase

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activity depending on the substrate. Through its interaction with Bα, E4orf4 may modulate the substrate specificity and/or subcellular localization of the Bα subset of PP2A enzyme.These results seem to indicate that the E4orf4-PP2A complex is required, but not sufficient to promote cell death.

5   Alternatively, some E4orf4 mutants may interact with Bα but fail to have a biological effect on PP2A due to inexact binding.

PP2A of *Saccharomyces (S.)cerevisiae* is very similar to the mammalian enzyme with respect to organization, substrate specificity and sensitivity to inhibitors. In yeast, the catalytic C subunit is encoded by *PPH21* and *PPH22*.

10   The products of *PPH21* and *PPH22* genes share 89% amino acid sequence identity and both are at least 75% identical to the human C subunit peptide sequences. Pph21 and Pph22 are unique in that, unlike PP2As of other species, they have acidic

amino terminal extensions of 60 and 68 residues, respectively. These stretches

15   share 48% identity. In *S. cerevisiae*, the catalytic subunits are dispensable for cell growth when deleted individually, while strains lacking both C subunits, as well as a related gene *PPH3*, are inviable.

TPD3 encodes the only A subunit, which like the mammalian protein, contains 15 non-identical repeats of 39 residues. Two B-type regulatory

20   subunits exist in yeast and are encoded by *CDC55* and *RTS1*, representing the B and B' families, respectively. *cdc55* strains are cold sensitive and produce cells that are abnormally elongated due to defects in cytokinesis and/or septation, implicating PP2A in cell morphogenesis. *rts1* strains show reduced growth at 30°C, while cells accumulate with a 2N DNA content at 37°C.

25   Neoplastic growth, or cancer, is the unregulated growth of cells and often leads to death. Surgical removal of the neoplastic cells can be ineffective because the tumor cells can invade normal, nondividing, tissue.

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Chemotherapy, another treatment, often causes severe side-effects in the patients. Thus there is a need for drugs and methods for the treatment of cancer that have greater efficacy and fewer side-effects than the current treatments.

5 ## Summary of the Invention

We have found a correlation between binding of E4orf4 to PP2A and E4orf4-mediated apoptosis. This association may be exploited for screening compounds, such as polypeptide or chemical compounds that bind to PP2A and selectively kill neoplastic cells, while not killing non-neoplastic cells, in

10 various *in vivo* and *in vitro* contexts. We have also discovered that the molecular interactions between E4orf4 and PP2A othologs are conserved in *S. cerevisiae*. Thus, *S. cerevisae* is useful for performing high-throughput screens for the identification of compounds that modulate or mimic E4orf4 interactions with PP2A.

15 Accordingly, in a first aspect, the invention features a method for identifying a compound as one useful for the treatment of cancer. The method includes the steps of (a) providing a first cell that has been modified to have no protein phosphatase 2A biological activity and a second cell that has not been modified to have no protein phosphatase 2A biological activity; (b) contacting

20 the cells with a candidate compound; and (c) assaying the cells for cell growth, wherein a decrease in cell growth of the second cell and no decrease in cell growth of the first cell identifies the candidate compound as a compound useful for the treatment of cancer.

In a second aspect, the invention features a second method for

25 identifying a compound as one useful for the treatment of cancer. This methodincludes the steps of (a) providing a first cell that has been modified to

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have no protein phosphatase 2A biological activity and a second cell that has not been modified to have no protein phosphatase 2A biological activity; (b) contacting the cells with a candidate compound; and (c) assaying the cells for cell death, wherein an increase in cell death of the second cell and no increase

5   in cell death of the first cell identifies the candidate compound as a compound useful for the treatment of cancer.

In a preferred embodiment of the first or second aspect, the first cell has a mutation in a gene encoding a subunit of protein phosphatase 2A that results in little or no protein phosphatase 2A biological activity. A preferred subunit

10   is a B subunit. Preferably, the cells are mammalian cells (e.g., human cells) or yeast cells (e.g., *S. cerevisiae* cells).

In a third aspect, the invention features a method of increasing cell death in a cell. The method includes the step of administering a compound that modulates protein phosphatase 2A (PP2A) biological activity. In one

15   preferred embodiment, the PP2A biological activity is increased. In another embodiment, the PP2A biological activity is decreased.

Preferably, the PP2A biological activity is dephosphorylation of a PP2A substrate. Exemplary PP2A substrates include, for example, Rb protein, Bcl-2, vimentin, casein-kinase II, and SR proteins.

20   In preferred embodiments, the compound is E4orf4 mRNA, a compound that increases stability of PP2A, an E4orf4 polypeptide (e.g., one that has been modified such that it has increased biological activity relative to an E4orf4 polypeptide that has not been modified), or has at least one E4orf4 biological activity.

25   In other preferred embodiments, the compound is a PP2A polypeptide (e.g., a PP2A polypeptide that has been modified such that it has increased biological activity relative to an PP2A polypeptide that has not been modified)

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or has at least one PP2A biological activity.

In a fourth aspect, the invention features another method of increasing apoptosis in a cell. The method includes administering to the cell an apoptosis-inducing amount of a PP2A polypeptide or cell death-inducing

5 fragment thereof.

In preferred embodiments of the third or fourth aspect, the cell is in a mammal, such as a human (e.g., a human having a cancer).

In a fifth aspect, the invention features a method of increasing apoptosis in a mammal. The method includes providing a transgene encoding a PP2A

10 polypeptide to a cell of the mammal, the transgene being positioned for expression in the cell. A preferred PP2A polypeptide includes a PP2A polypeptide that has been modified such that it has increased biological activity relative to an PP2A polypeptide that has not been modified or a PP2A polypeptide that has at least one PP2A biological activity.

15 In a sixth aspect, the invention features a pharmaceutical composition that includes (i) substantially pure nucleic acid encoding a PP2A polypeptide or a cell death-inducing fragment thereof; and (ii) a pharmaceutically acceptable carrier, the composition useful for the treatment of cancer.

In a seventh aspect, the invention features a pharmaceutical composition

20 that includes (i) substantially pure mammalian PP2A polypeptide or a cell death-inducing fragment thereof; and (ii) a pharmaceutically acceptable carrier, the composition useful for the treatment of cancer.

In an eighth aspect, the invention features a pharmaceutical composition that includes (i) a therapeutic amount of a compound that modulates PP2A

25 biological activity; and (ii) a pharmaceutically acceptable carrier, the composition useful for the treatment of cancer.

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In a ninth aspect, the invention features a pharmaceutical composition that includes (i) a therapeutic amount of a compound that binds to PP2A; and (ii) a pharmaceutically acceptable carrier, the composition useful for the treatment of cancer.

5   In preferred embodiments of the eighth or ninth aspect, the compound is an E4orf4 polypeptide that has been modified such that it has increased biological activity relative to an E4orf4 polypeptide that has not been modified, an agonist of E4orf4, or mimics at least one E4orf4 biological activity.

10   In a tenth aspect, the invention features a method for identifying a compound useful for the treatment of cancer. The method includes the steps of: (a) providing a cell expressing PP2A; (b) contacting the cell with a candidate compound; and (c) measuring binding of the compound to PP2A, wherein the binding of the compound to the PP2A indicating a compound that

15   is useful for the treatment of cancer.

In an eleventh aspect, the invention features another method for identifying a compound useful for the treatment of cancer. The method includes the steps of: (a) providing a cell expressing PP2A; (b) contacting the cell with a candidate compound; and (c) measuring biological activity of the

20   PP2A in the cell, wherein a change in the biological activity relative to a cell not contacted with the candidate compound indicating a compound that is useful for the treatment of cancer.

In preferred embodiments of the tenth or eleventh aspect, the cell is a mammalian cell or a yeast cell (e.g., *S. cerevisiae*).

25   In a twelfth aspect, the invention features another method for identifying a compound useful for the treatment of cancer. The method includes the steps of: (a) contacting a candidate compound with PP2A; and (b)

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measuring binding of the compound to PP2A, wherein a change in the binding indicating a compound that is useful for the treatment of cancer.

In a thirteenth aspect, the invention features yet another method for identifying a compound useful for the treatment of cancer. The method

5   includes the steps of: (a) contacting a candidate compound with PP2A; and (b) measuring biological activity of the PP2A in the cell, wherein a change in the biological activity relative to a cell not contacted with the candidate compound indicating a compound that is useful for the treatment of cancer.

In a fourteenth aspect, the invention features a method for treating a

10   human having abnormal or excessive cell growth. The method includes administering a compound that mimics or modulates PP2A biological activity.

In a fifteenth aspect, the invention features another method for treating a human having abnormal or excessive cell growth, the method includes administering a compound that binds to PP2A.

15   In a sixteenth aspect, the invention features another method for identifying a compound as one useful for the treatment of cancer. The method includes: (a) providing a cell capable of expressing PP2A; (b) contacting the cell with a first compound that decreases PP2A biological activity in the cell; (c) contacting the cell with a second compound; and (d) assaying cell death of

20   the cell, wherein a decrease in cell death relative to the cell contacted with the second compound but not the first compound identifies the second compound as a compound useful for the treatment of cancer.

In a seventeenth aspect, the invention features another method for identifying a compound as one useful for the treatment of cancer. This method

25   includes: (a) providing a cell capable of expressing PP2A; (b) expressing a first compound that decreases PP2A biological activity in the cell; (c) contacting the cell with a second compound; and (d) assaying cell death of the

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cell, wherein a decrease in cell death relative to the cell contacted with the second compound but not expressing the first compound identifies the second compound as a compound useful for the treatment of cancer.

In a preferred embodiment of the sixteenth or seventeenth aspect, the
5   first compound is an E4orf4 nucleic acid (e.g., a sense or antisense E4orf4 nucleic acid that is operably linked to an inducible promoter). In another preferred embodiment, the first compound is a PP2A A subunit that does not bind to a PP2A B subunit. Preferably, the cell contains nucleic acid sequence, encoding the PP2A A subunit that does not bind to a PP2A B subunit, that is
10   operably linked to an inducible promoter.

In still another preferred embodiment of the sixteenth or seventeenth aspect, the first compound is a polypeptide selected from the group of consisting of polyomavirus small T antigen, polyomavirus middle T antigen, and SV40 virus small T antigen. Preferably, the cell contains nucleic acid
15   sequence, encoding polypeptide, that is operably linked to an inducible promoter.

In an eighteenth aspect, the invention features still another method for identifying a compound as one useful for the treatment of cancer. This method includes: (a) providing a cell that expresses an E4orf4 polypeptide that binds to
20   a PP2A subunit but does not kill the cell; (b) contacting the cell with a compound; and (c) assaying cell death of the cell, wherein a decrease in cell death relative to a cell not expressing the E4orf4 polypeptide identifies the compound as a compound useful for the treatment of cancer.

In a nineteenth aspect, the invention features another method for
25   identifying a compound as one useful for the treatment of cancer. This method includes: (a) providing a protein complex that comprises Bα subunit of PP2A associated with an E4orf4 polypeptide; (b) exposing the protein complex to a

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candidate compound; and (c) assaying the amount of the E4orf4 polypeptide that is not associated with the Bα subunit of PP2A, wherein an increase in the amount of the E4orf4 polypeptide that is not associated with the Bα subunit of PP2A identifies the candidate compound as a compound useful for the

5   treatment of cancer.

In a preferred embodiment, the protein complex is attached to a solid support. Preferably, the Bα subunit of PP2A or the E4orf4 polypeptide is detectably labeled.

By a "substantially pure protein" is meant a protein of the invention that

10   has been separated from components which naturally accompany it. Typically, the protein is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a

15   polypeptide of the invention. A substantially pure protein of the invention may be obtained, for example, by extraction from a natural source (for example, a virus), by expression of a recombinant nucleic acid encoding such a protein, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel

20   electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be

25   substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

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By "substantially pure nucleic acid" is meant a nucleic acid (e.g., DNA) that is free of the genes, or transcripts thereof, which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA

5   which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule which is

10   transcribed from a DNA molecule, as well as a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "E4orf4 protein" is meant a polypeptide product encoded by nucleic acid capable of hybridizing at high stringency conditions to a nucleic acid molecule encoding E4orf4 and having at least one E4orf4 biological activity.

15   It will be understood that E4orf4 proteins and nucleic acids of the invention may be obtained from any adenovirus strain having the E4orf4 open reading frame, as defined as an open reading frame which is at least 20%, preferably 50%, more preferably 75%, and most preferably 90% identity to the E4orf4 open reading frame of adenovirus serotype Ad2, Ad5, Ad12, or Ad40.

20   By "PP2A" or "protein phosphatase 2A" is meant a polypeptide product encoded by nucleic acid capable of hybridizing at high stringency conditions to a nucleic acid molecule encoding a PP2A subunit which, when combined with other subunits, has at least one PP2A biological activity. A preferred PP2A biological activity is dephosphorylation of a PP2A substrate. PP2A consists of

25   three subunits (A, B, and C). It will be understood that PP2A proteins and nucleic acids of the invention may be obtained from any source having a PP2A open reading frame, as defined as an open reading frame which is at least 20%,

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preferably 50%, more preferably 75%, and most preferably 90% identity to the PP2A open reading frame of human PP2A or *S. cerevisiae CDC55.*

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.,* Sequence Analysis

5 Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). Most preferably, the E4orf4 protein has the consensus sequence shown in Fig. 1, where X can be any amino acid residue.

10 By "high stringency conditions" is meant conditions that allow DNA hybridization to nucleic acids encoding E4orf4 at high stringency (*e.g.,* hybridizing in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides). For other definitions of high stringency conditions, see Ausubel, F. et al., 1994, <u>Current Protocols in Molecular Biology</u>, John Wiley & Sons,

15 New York, 6.3.1 - 6.3.6, hereby incorporated by reference.

By "E4orf4 biological activity" is meant binding to the B subunit of PP2A, modulating PP2A biological activity, and specifically inducing, in tumor cells, biological processes that result in cell death.

By "PP2A biological activity" is meant dephosphorylation of serine or

20 threonine residues of PP2A substrates. PP2A substrates include, for example, Rb protein, Bcl-2, vimentin, casein-kinase II, and SR proteins. Those in the art will recognize that there are other PP2A substrates.

By "interact" or "bind" is meant that two polypeptides can transitorily bind, either directly or indirectly, to each other. Indirect binding can take

25 place, for example, through the formation of a multi-protein complex. The interaction is dependent on inherent properties of the two polypeptides, and specifically excludes non-specific (e.g., strictly Brownian) contact. Preferably,

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this interaction can be disrupted or prevented by alteration in the primary, secondary, or tertiary structure of one or both of the polypeptides.

By "expose" is meant to allow contact between an animal, cell, lysate, or extract derived from a cell, or molecule derived from a cell, and a candidate

5   compound.

By "treat" is meant to submit or subject an animal (e.g. a human), cell, lysate or extract derived from a cell, or molecule derived from a cell to a candidate compound.

By "candidate compound" is meant a chemical, be it naturally-occurring

10   or artificially-derived, that is assayed for its ability to modulate an alteration in reporter gene activity or protein levels, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

15   By "assaying" is meant analyzing the effect of a treatment, be it chemical or physical, administered to whole animals or cells derived therefrom. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be, for example, for the purpose of detecting altered gene expression, altered RNA

20   stability, altered protein stability, altered protein levels, or altered protein biological activity. The means for analyzing may include, for example, antibody labeling, immunoprecipitation, phosphorylation assays, and methods known to those skilled in the art for detecting nucleic acids and polypeptides.

By "modulating" is meant changing, either by decrease or increase,

25   biological activity.

By "a decrease" is meant a lowering in the level of biological activity, as measured by a lowering of (i) PP2A protein, as measured by ELISA;

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(ii) PP2A phosphatase activity, as measured, for example, by standard techniques; (iii) PP2A mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (iv) the amount of

5   E4orf4 (or any other protein that is part of a protein complex that contains E4orf4) that interact with PP2A; and (v) PP2A-mediated cell killing. In all cases, the lowering is preferably by 20%, more preferably by 40%, and even more preferably by 70%.

By "an increase" is meant a rise in the level of biological activity, as

10   measured by an increase of: (i) PP2A protein, as measured by ELISA;

(ii) PP2A phosphatase activity, as measured, for example, by standard techniques; (iii) PP2A mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (iv) the amount of

15   E4orf4 (or any other protein that is part of a protein complex that contains E4orf4) that interact with PP2A; and (v) PP2A-mediated cell killing. Preferably, the increase is by 5% or more, more preferably by 15% or more, even more preferably by 2-fold, and most preferably by at least 3-fold.

By "transfection" is meant any method for introducing foreign

20   molecules into a cell. Lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the teachings which may be used.

By "pharmaceutically acceptable carrier" is meant a carrier that is physiologically acceptable to the treated mammal while retaining the

25   therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one

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skilled in the art and described, for example, in <u>Remington: The Science and Practice of Pharmacy</u>, (19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA.

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic illustration showing alignment of E4orf4 proteins from adenovirus serotypes (Ad5, Ad9, Ad12, and Ad40). The consensus sequence of adenovirus E4orf4 is shown as the top sequence.

Fig. 2 is a schematic illustration of the various residues that were point mutated in Ad2 serotype E4orf4. Each of the indicated residues was mutated to alanine, such that, for example, the P4 mutant has an alanine residue at position 4 instead of proline.

Fig. 3 is a photograph of an anti-HA Western blotting analysis showing the expression of the Ad2 E4orf4 point mutants (and wild-type controls) in whole cell extracts (WCE) of C33A cells transfected with the hemagglutinin (HA)-tagged Ad2 E4orf4 point mutants.

Fig. 4A is a schematic illustration depicting the PP2A/E4orf4 complex. Note the hemagglutinin (HA) tag on E4orf4 and the Flag-tag on the Bα subunit of PP2A.

Fig. 4B is a schematic diagram of the steps involved in the immunoprecipitation of HA-tagged E4orf4, followed by an *in vitro*

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phosphatase assay; a whole cell extract followed by immunoblotting; or a drug resistant colony assay, following the co-transfection of cells with an HA-tagged E4orf4 mutant a Flag-tagged Bα subunit of PP2A.

Fig. 4C is a schematic illustration showing the results of the *in vitro*
5   phosphatase assay following HA-tagged E4orf4 immunoprecipitation from cells co-transfected with Flag-tagged Bα subunit of PP2A and the indicated HA-tagged E4orf4 mutant (or mock transfected).

Fig. 4D is a photograph of Western blotting analysis of the SDS-PAGE resolved samples that were assayed for *in vitro* phosphatase activity in the
10   experiment shown in Fig. 4C (E4orf4 IP lane) using an anti-Flag antibody, which detected the Flag-tagged PP2A Bα subunit. Below (the WCE lane) is a Western blotting analysis of whole cell extracts from transfected cells.

Fig. 5 is a schematic illustration of the areas in E4orf4 that are important for binding to PP2A Bα subunit and/or important for suppressing colony
15   formation of transfected cells. These areas are shown in diagram of the linear E4orf4 amino acid sequence. Areas in solid black bars indicate the areas of E4orf4 which, when mutated, result in a loss of binding of E4orf4 to PP2A Bα subunit and a loss of phosphatase activity of PP2A (which is lost since it is no longer associated with these E4orf4 mutants), and do not result in any
20   suppression of colony formation of cells stably transfected with the E4orf4 mutant. Areas in diagonal hatch bars indicate the areas of E4orf4 that, when mutated, result in a partial loss of binding of E4orf4 to PP2A Bα subunit, a partial loss of phosphatase activity of associated PP2A, and a partial suppression of colony formation of stably transfected cells. Areas in horizontal
25   hatch bars indicate the areas of E4orf4 that, when mutated, result in a normal (wild-type) binding of E4orf4 to PP2A Bα subunit, wild-type levels of phosphatase activity of associated PP2A, and a partial suppression of colony

formation of stably transfected cells.

Fig. 6 is a schematic illustration of relative cell death of indicated cell lines stably transfected with an inactive E4orf4 mutant (white bars outlined in black), wild-type E4orf4 (black bars), or Bax (stippled bars).

5   Fig. 7 is a schematic illustration how the tetracycline-inducible E4orf4-encoding virus (Ad5-TindHAorf4) was constructed. Also shown in this diagram is the construction strategy used to generate a virus expressing the reverse tet-transactivator operably linked to the CMV promoter (Ad5-CMVrtTA).

10   Fig. 8 is a photograph of an immunoblot showing inducible E4orf4 expression with the Ad5-TindHAorf4 virus. Mrc-5, HeLa, or C33A cells were infected (+) with Ad5-TindHAorf4 and Ad5-CMVrtTA. HA-tagged E4orf4 (detected by anti-HA antibody) was induced by addition to the infected cells of 10 μg/ml doxycycline.

15   Fig. 9 is a series of photographs of Western blots showing the dose- and temporal-response to doxycycline.

Fig. 10 is a schematic illustration showing that E4orf4 inhibits growth of H1299 lung carcinoma cells.

Fig. 11 is a series of schematic illustrations showing that E4orf4 inhibits

20   growth of H1299 and C33A tumor cell lines.

Fig. 12 is a series of schematic illustrations showing that E4orf4 does not inhibit growth of HMEC or NHDF cells, two normal primary cells.

Figs. 13A and 13B are a series of photographs showing the effect of E4orf4 protein expression on yeast colony growth.

25   Figs. 14A and 14B are a series of photographs showing that yeast expressing E4orf4 are viable. Haploid yeast containing either vector alone or HA-E4orf4 were grown in glucose-based medium. Cells were transferred to

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glucose or galactose and aliquots of cells were removed at various times post-induction and the percentage of cells excluding trypan blue was determined (Fig. 14A). Whole cell extracts were prepared from cells collected at the indicated times and subject to immunoblot assay using anti-HA antibody
5   (Fig. 14B).

     Fig. 15A-15C are a series of photographs showing that E4orf4 expression in wild-type yeast confers an elongated cell morphology. Yeast transformed with vector alone (Fig. 15A) or E4orf4 (Fig. 15B) were cultured in galactose-based medium at 30?C. The cdc55 strain was grown in galactose
10   medium at 18?C (Fig. 15C). Figs. 16A and 16B are a serues of photographs showing that E4orf4 requires Cdc55 to induce the slow growth phenotype. Wild-type and cdc55 cells were transformed with vector or E4orf4. Yeast were serially diluted and spotted onto glucose- or galactose-based agar plates. Photographs were taken at day 3 post-induction (Fig. 16A). Whole cell
15   extracts prepared from yeast cells collected from the above plates were subject to SDS-PAGE immunoblot assay using anti-HA antibody (Fig. 16B). Lanes 1 and 3 represent vector alone and lanes 2 and 4 represent E4orf4 expression.

     Figs. 17A-17F are a series of photographs showing that E4orf4 requires Cdc55 to recruit the PP2A complex. The cdc55 strain was co-transformed with
20   the indicated plasmids (pYES2, p424GAL1, pYes2-HAE4orf4 or p424GAL-FlagCDC55) and whole cell extracts were prepared 24 hours post-galactose induction. Extracts were incubated with anti-HA or anti-Flag antibodies. Immunoprecipitates were separated by SDS-PAGE and subject to immunoblotting with anti-HA (Figs. 17A and 17D), anti-Flag (Figs. 17B and
25   17E) or rabbit polyclonal anti-Tpd3 antibodies (Figs. 17C and 17F).

     Figs. 18A-18I are a series of photographs showing E4orf4 can immunoprecipitate PP2A complexes containing both forms of the yeast

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catalytic subunit.   1. v+v; 2. v+PPH21; 3. v+PPH22; 4. E4orf4+v; 5.
E4orf4+PPH21; 6. E4orf4+PPH22.   Wild-type yeast were co-transformed with
plasmids expressing the indicated proteins and cells were collected 24 hours
post-galactose induction.   Whole cell extracts (wce) were prepared and subject

5   to immunoprecipitation with antibody against the Flag or HA epitope.   Proteins
were separated by SDS PAGE followed by Western blotting with anti-HA
(Figs. 18A and 18D), anti-Flag (Figs. 18B and 18E), or anti-Tpd3 antibodies
(Figs. 18C and 18E).   Protein expression from wce was also determined (Figs.
18G-18I).

10   Figs. 19A-19C are a series of photographs showing analysis of colony
growth and Cdc55 binding using E4orf4 point mutants.   Fig. 19A shows yeast
transformed with plasmids expressing the indicated HA-tagged proteins and
spotted onto glucose- or galactose-based medium and incubated at 30?C.
Pictures were taken at day 3.   Fig. 19B shows anti-HA western blot of whole

15   cell extracts prepared from cells expressing the indicated proteins.   Fig. 19C
shows co-immunoprecipitation experiments using whole cell extracts prepared
from the cdc55 strain transformed with the indicated plasmids; CDC55 is
Flag-tagged.

            Figs. 20A and 20B are a series of photographs showing that the slow

20   growth effect of E4orf4 in yeast specifically requires CDC55 and not RTS1.
In Fig. 20A, wild-type or RTS1 deleted cells were transformed with vector,
E4orf4 or E4orf4 point mutants.   Serial dilutions of cultured cells were spotted
onto glucose or galactose-based agar plates, and photographs were taken on
day 3 post-incubation.   Fig. 20B shows whole cell extracts immunoblotted for

25   the HA-epitope to confirm protein expression.

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Detailed Description of the Invention

We have found a correlation between binding of E4orf4 to PP2A and E4orf4-mediated apoptosis. For example, we have discovered that E4orf4 selectively associates with PP2A. Thus, an E4orf4 mutant having an ability to
5 more tightly associate with PP2A Bα subunit may be able to induce a more rapid cell death in a neoplastic cell (*e.g.*, C33A) while not killing a non-neoplastic cell (*e.g.*, Mrc-5). This association may be exploited for screening compounds, such as polypeptide or chemical compounds that bind to PP2A and selectively kill neoplastic cells, while not killing non-neoplastic cells, in
10 various *in vivo* and *in vitro* contexts. Mutant PP2A proteins or PP2A mimetics may also be developed, using the teachings described herein, which have an enhanced ability to kill neoplastic cells, while retaining an inability to kill normal cells.

We have also discovered that the molecular interactions between E4orf4
15 and PP2A are conserved in *S. cerevisiae*. Thus, *S. cerevisae* is useful for performing high-throughput screens for the identification of compounds that modulate or mimic E4orf4 interactions with PP2A.

The E4orf4 proteins, mutants, or mimetics thereof of the invention, may be recombinantly produced in a cell transfected with an expression plasmid.
20 Alternatively, the invention provides substantially purified DNA encoding E4orf4 proteins, mutants, or analogs thereof. In addition, the invention provides a vector (*e.g.*, an adenoviral vector) encoding an E4orf4 protein (or mutant thereof). The viruses lacking an E1B-19K protein, described in U.S. Patent No. 5,667,178, are specifically excluded from the present invention.
25 Preferably, the vector is genetically engineered.

It will be understood that the E4orf4-encoding adenoviruses of the invention may be manipulated using the techniques and assays provided in

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U.S. Patent No. 5,667,178 (issued October 14, 1997) or PCT Application No.
PCT IB97/01041 (published January 15, 1998), both of which are incorporated
by reference.

Production of E4orf4 and PP2A polypeptides

5   For protein expression, eukaryotic and prokaryotic expression systems
may be generated in which nucleic acid sequences are introduced into a
plasmid or other vector, which is then used to transform living cells.
Constructs in which cDNA containing the entire open reading frames encoding
E4orf4 or a PP2A subunit, inserted in the correct orientation into an expression

10   plasmid, may be used for protein expression. Alternatively, portions of the
nucleic acid sequences, including those encoding wild-type or mutant E4orf4
or PP2A subunits, may be inserted. Prokaryotic and eukaryotic expression
systems allow various important functional domains of the proteins to be
recovered as fusion proteins and then used for binding, structural and

15   functional studies and also for the generation of appropriate antibodies. Since
expression of E4orf4 and PP2A polypeptides may induce cell death in some
cell types (e.g., tumor cells), it may be desirable to express the protein under
the control of an inducible promoter, as described herein.

         Typical expression vectors contain promoters that direct the synthesis of

20   large amounts of mRNA corresponding to the inserted nucleic acid in the
plasmid bearing cells. They may also include eukaryotic or prokaryotic origin
of replication sequences which allow for their autonomous replication within
the host organism, sequences that encode genetic traits that allow vector-
containing cells to be selected for in the presence of otherwise toxic drugs, and

25   sequences that increase the efficiency with which the synthesized mRNA is
translated. Stable long-term vectors may be maintained as freely replicating

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entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced which have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

5   Expression of foreign sequences in bacteria, such as *Escherichia coli*, requires the insertion of the nucleic acid sequence into a bacterial expression vector. This plasmid vector contains several elements required for the propagation of the plasmid in bacteria, and expression of inserted DNA of the plasmid by the plasmid-carrying bacteria. Propagation of only plasmid-bearing

10   bacteria is achieved by introducing in the plasmid selectable marker-encoding sequences that allow plasmid-bearing bacteria to grow in the presence of otherwise toxic drugs. The plasmid also bears a transcriptional promoter capable of producing large amounts of mRNA from the cloned gene. Such promoters may or may not be inducible promoters, which initiate transcription

15   upon induction. The plasmid also preferably contains a polylinker to simplify insertion of the gene in the correct orientation within the vector. In a simple *E. coli* expression vector utilizing the lac promoter, the expression vector plasmid contains a fragment of the *E. coli* chromosome containing the lac promoter and the neighboring *lacZ* gene. In the presence of the lactose analog IPTG, RNA

20   polymerase normally transcribes the *lacZ* gene, producing *lacZ* mRNA, which is translated into the encoded protein, β-galactosidase. The *lacZ* gene can be cut out of the expression vector with restriction endonucleases and replaced by an E4orf4 or PP2A nucleic acid sequence, or fragment, fusion, or mutant thereof. When this resulting plasmid is transfected into *E. coli*, addition of

25   IPTG and subsequent transcription from the lac promoter produces mRNA, which is translated into an E4orf4 or PP2A polypeptide.

Once the appropriate expression vectors containing an E4orf4 or PP2A gene, or fragment, fusion, or mutant thereof, are constructed, they are introduced into an appropriate host cell by transformation techniques including, for example, calcium phosphate transfection, DEAE-dextran

5   transfection, electroporation, microinjection, protoplast fusion and liposome-mediated transfection, as described herein. The host cells that are transfected with the vectors of this invention may include (but are not limited to) *E. coli*, *Pseudomonas, Bacillus subtilus*, or other bacilli, other bacteria, yeast, fungi, insect (using, for example, baculoviral vectors for expression), mouse or other

10   animal or human tissue cells. Mammalian cells can also be used to express protein using a vaccinia virus expression system described in Ausubel et al., 1997, Current Protocols in Molecular Biology, Wiley Interscience, New York.

In vitro expression of E4orf4 and PP2A proteins, fusions, polypeptide fragments, or mutants encoded by cloned DNA is also possible using the T7

15   late-promoter expression system. This system depends on the regulated expression of T7 RNA polymerase which is an enzyme encoded in the DNA of bacteriophage T7. The T7 RNA polymerase transcribes DNA beginning within a specific 23-bp promoter sequence called the T7 late promoter. Copies of the T7 late promoter are located at several sites on the T7 genome, but none

20   is present in *E. coli* chromosomal DNA. As a result, in T7 infected cells, T7 RNA polymerase catalyzes transcription of viral genes but not of *E. coli* genes. In this expression system recombinant *E. coli* cells are first engineered to carry the gene encoding T7 RNA polymerase next to the lac promoter. In the presence of IPTG, these cells transcribe the T7 polymerase gene at a high rate

25   and synthesize abundant amounts of T7 RNA polymerase. These cells are then transformed with plasmid vectors that carry a copy of the T7 late promoter protein. When IPTG is added to the culture medium containing these

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transformed *E. coli* cells, large amounts of T7 RNA polymerase are produced. The polymerase then binds to the T7 late promoter on the plasmid expression vectors, catalyzing transcription of the inserted cDNA at a high rate. Since each *E. coli* cell contains many copies of the expression vector, large amounts

5   of mRNA corresponding to the cloned cDNA can be produced in this system and the resulting protein can be radioactively labeled. Plasmid vectors containing late promoters and the corresponding RNA polymerases from related bacteriophages such as T3, T5, and SP6 may also be used for *in vitro* production of proteins from cloned DNA. *E. coli* can also be used for

10   expression using an M13 phage such as mGPI-2. Furthermore, vectors that contain phage lambda regulatory sequences, or vectors that direct the expression of fusion proteins, for example, a maltose-binding protein fusion protein or a glutathione-S-transferase fusion protein, also may be used for expression in *E. coli*.

15   Eukaryotic expression systems permit appropriate post-translational modifications to expressed proteins. Transient transfection of a eukaryotic expression plasmid allows the transient production of a polypeptide by a transfected host cell. E4orf4 and PP2A proteins may also be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable

20   transfection of mammalian cells are available to the public (e.g., see Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, Supp. 1987 and VectorDB at URL address http://vectordb.atcg.com/), as are methods for constructing such cell lines (see e.g., Ausubel et al., *supra*). In one example, cDNA encoding an E4orf4 or PP2A protein, fusion, mutant, or polypeptide fragment

25   is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the protein-encoding nucleic acid sequence into the host cell chromosome is

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selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., *supra*). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods

5   for selecting cell lines bearing gene amplifications are described in Ausubel et al., *supra*. These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors are pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). The host cells described

10   above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among those most preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Eukaryotic cell expression of E4orf4 and PP2A proteins allows the

15   production of large amounts of normal and mutant proteins for isolation and purification, and the use of cells expressing these proteins as a functional assay system for antibodies generated against the protein. Eukaryotic cells expressing one or both of these proteins may also be used to test the effectiveness of pharmacological agents on E4orf4/PP2A-mediated biological

20   activity (e.g., killing of tumor cells), or as means by which to study E4orf4 and PP2A proteins as components of a signal transduction pathway. Expression of proteins, fusions, mutants, and polypeptide fragments in eukaryotic cells also enables the study of the function of the normal complete protein, specific portions of the protein, or of naturally occurring polymorphisms and artificially

25   produced mutated proteins. E4orf4 and PP2A DNA sequences can be altered using procedures known in the art, such as restriction endonuclease digestion, DNA polymerase fill-in, exonuclease deletion, terminal deoxynucleotide

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transferase extension, ligation of synthetic or cloned DNA sequences and site-directed sequence alteration using specific oligonucleotides together with PCR.

Another preferred eukaryotic expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from

5   Clontech (Palo Alto, CA). If desired, this system may be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (Mol. Cell Biol. 5:3610-3616, 1985).

Once the recombinant protein is expressed, it can be isolated from the expressing cells by cell lysis followed by protein purification techniques, such

10   as affinity chromatography. For example, an anti-E4orf4 antibody can be attached to a column and used to isolate the recombinant E4orf4 proteins. Lysis and fractionation of E4orf4 protein-harboring cells prior to affinity chromatography may be performed by standard methods (see e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be purified

15   further by e.g., by high performance liquid chromatography (HPLC; e.g., see Fisher, <u>Laboratory Techniques In Biochemistry And Molecular Biology</u>, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short E4orf4 fragments and longer fragments of the N-terminus and C-terminus of the E4orf4 protein, can

20   also be produced by chemical synthesis (e.g., by the methods described in <u>Solid Phase Peptide Synthesis</u>, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful E4orf4 polypeptide fragments or analogs, as described herein.

25   Those skilled in the art of molecular biology will understand that a wide variety of expression systems may be used to produce the recombinant E4orf4 and PP2A proteins. The precise host cell used is not critical to the invention.

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The proteins may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells such as Sf9 cells, or mammalian cells such as COS-1, NIH 3T3, or HeLa cells). These cells are commercially available from, for example, the American Type Culture Collection, Rockville,

5   MD (see also Ausubel et al., *supra*). The method of transformation and the choice of expression vehicle (e.g., expression vector) will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., *supra*, and expression vehicles may be chosen from those provided, e.g. in Pouwels et al., *supra*.

10   <u>Testing for the presence of E4orf4 and PP2A biological activity</u>

Identification of both biologically active and mutant forms of E4orf4 allows the study of E4orf4 biological activity. For example, administration of an E4orf4 protein, or a polypeptide fragment thereof, may have an ability to modulate PP2A biological activity (e.g., phosphatase activity), as measured by

15   cell-based and cell-free assays known in the art and described herein, and/or kill tumor cells. Such assays may be carried out in a cell which either expresses endogenous PP2A or a cell to which is introduced a heterologous amount of a PP2A polypeptide or in a cell-free assay. Preferably, the cell is capable of undergoing E4orf4-mediated cell death.

20   One preferred E4orf4 biological activity is binding of E4orf4 to the B subunit of PP2A. It is understood that the binding may not be through direct contact between E4orf4 and PP2A, but may require additional proteins. Binding of E4orf4 to PP2A, or any other protein that is part of a protein complex that contains PP2A, can be measured using any of a number of

25   standard methods known to those in the art, such as the methods described herein.

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Another preferred E4orf4 biological activity is the specific killing of tumor cells. The killing of cells is very likely to require binding to PP2A. Thus a compound that binds to PP2A should also be screened for its ability to kill tumor cells.

5 ## Identification of molecules that modulate or mimic E4orf4 or PP2A biological activity

Methods of observing changes in E4orf4- or PP2A-mediated biological activity, such as the ones described above, are exploited in high-throughput assays for the purpose of identifying compounds that modulate specific killing

10 of tumor cells. Compounds that mime E4orf4 or PP2A activity also may be identified by such assays. Such identified compounds may have utility as therapeutic agents in the treatment of cell proliferation disease.

In general, novel drugs that mimic E4orf4 biological activity or modulate or mimic PP2A biological activity are identified from large libraries

15 of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or

20 compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed

25 synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and

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nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are

5   commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation

10 methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any

15 combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for cell proliferation diseases should be employed whenever possible.

When a crude extract is found to mimic E4orf4 biological activity or modulate or mimic PP2A biological activity, further fractionation of the

20 positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that mimic E4orf4 biological activity or modulate or mimic PP2A biological activity. The same assays described

25 herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the

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art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using a standard animal or culture model for a cell proliferative disease known in the

5   art.

The effect of candidate compounds on E4orf4/PP2A-mediated regulation of cell death may be measured at the level of PP2A phosphatase activity by using the general approach described above with standard protein detection techniques, such as Western blotting or immunoprecipitation with a

10   PP2A-specific antibody.

Compounds that mimic E4orf4 biological activity or modulate or mimic PP2A biological activity may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel et al., *supra*). In an assay of a mixture of

15   compounds, E4orf4 or PP2A biological activity is measured in cells administered progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to mimic E4orf4 biological activity or modulate or mimic PP2A biological

20   activity. The degree of biological activity in the presence of a candidate compound is compared to the degree of biological activity in its absence, under equivalent conditions. Biological activity may be measured by any standard assay, for example, those described herein.

Another method for detecting compounds that modulate the activity of

25   PP2A is to screen for compounds that bind to PP2A. These compounds may be detected by adapting interaction trap expression systems known in the art. These systems detect protein interactions using a transcriptional activation

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assay and are generally described by Gyuris et al. (Cell 75:791-803, 1993) and Field et al., (Nature 340:245-246, 1989), and are commercially available from Clontech (Palo Alto, CA). Alternatively, compounds that bind to PP2A can be isolated by column chromatography. In this assay, candidate compounds or

5   extracts are passed through a column containing immobilized PP2A B subunit or PP2A holoprotein. After washing to remove nonspecific proteins, the compounds bound to PP2A are eluted, collected, and identified. Using similar assays, compounds that interfere with PP2A binding to E4orf4 can also be identified.

10   As described herein, we have now shown that it is very highly likely that E4orf4-mediated killing of tumor cells requires binding to PP2A; every E4orf4 mutant that is capable of killing also binds. In one embodiment, an E4orf4 analog is one that cannot kill (or exhibits a decrease in killing) in the absence of PP2A biological activity. Those in the art will recognize that any

15   strategy that decreases PP2A biological activity is useful in the present invention.

We identified E4orf4 polypeptides that are partially defective for colony suppression, but wild-type for binding to PP2A. These polypeptides as dominant-negative E4orf4 polypeptides and are useful as competitive reagents

20   for identifying compounds that kill by binding to PP2A in a manner similar to that of PP2A.

Thus, one series of assays include determining the ability the compounds, known to be toxic, to kill cells expressing a dominant-negative E4orf4 polypeptide. Dominant-negative PP2A polypeptides can also be made.

25   For example, PP2A A subunit mutants can be made which fail to interact with specific B subunits, but which still bind to the catalytic C subunit. Finally, the small T antigens of polyomavirus and SV40 virus and the middle T antigen of

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polyomavirus interact with the PP2A-A subunit, also inhibiting interactions with the B subunit, which is the likely mechanism of E4orf4/PP2A-mediated killing of tumor cells. Overexpression of one of these A subunit mutants, small T antigen, or middle T antigen would lead to PP2A lacking the B

5   subunit; this would inhibit the formation of E4orf4/PP2A holoenzyme complex, and would likely inhibit E4orf4-mediated killing.

In a related approach, a cell line that lacks functional PP2A Bα subunit can be made using standard homologous recombination techniques. It is likely that generation of a mouse lacking PP2A Bα subunit will not be viable, but cell

10   lines derived from embryonic stem cells may be produced. If necessary, the deleted gene can be complemented with a copy of the gene under the control of an inducible promoter, such that cells may be grown in the presence of functional enzyme, but the assays performed in the absence of functional enzyme.

15   Antisense based strategies may be employed to reduce PP2A biological activity. The principle is based on the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the

20   processing/transport/translation and/or stability of the target PP2A mRNA. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides and injection of antisense RNA. Antisense effects can be induced by control (sense) sequences, however, the extent of phenotypic changes are highly variable. Phenotypic effects induced by

25   antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

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Reduction of PP2A biological activity may be accomplished by direct administration of antisense PP2A mRNA to a cell in which proliferation is desired. The antisense PP2A mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using

5   an antisense PP2A cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense PP2A mRNA to cells can be carried out by any of the methods for direct nucleic acid administration described above.

An alternative strategy for decreasing PP2A biological activity involves

10 intracellular expression of an anti-PP2A antibody or a portion of an anti-PP2A antibody. For example, a nucleic acid (or fragment thereof) encoding a monoclonal antibody that specifically binds to PP2A and inhibits its biological activity may be placed under the transcriptional control of a cell type-specific gene regulatory sequence.

15   Most likely, PP2A is actively involved in the cell killing, by causing one or more dephosphorylation events. This may be due to an increase in overall PP2A phosphatase activity such that all PP2A substrates are dephosphorylated at an increased rate. Alternatively, it may be due to that one or a subset of PP2A substrates are dephosphorylated at an increased rate. This second,

20 selective, increase may be due to a conformational change in the PP2A holoenzyme, leading to enhanced enzyme-substrate interactions. Alternatively, E4orf4 may target PP2A to specific substrates by altering the subcellular localization of PP2A. In each scenario, a death-inducing protein could be activated, or a survival factor could be inactivated, by dephosphorylation.

25   Rather than activating dephosphorylation, E4orf4, by binding to PP2A, may interfere with the ability of PP2A to perform a dephosphorylation function. Several different mechanisms could account for such interference:(i)

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E4orf4 could alter the subcellular localization of PP2A, making it no longer accessible to a substrate; (ii) a substrate could be displaced by E4orf4, or its binding site could be blocked; or (iii) E4orf4 binding to PP2A could induce a conformational change in PP2A that reduces its affinity for a substrate. In each of the three cases, the interference could be generally to all PP2A substrates, or it could be restricted to one or a subset of PP2A substrates.

Several pathways are affected by E4orf4/PP2A interactions. E4orf4/PP2A interaction causes alterations in the activity of several transcriptions, including, for example, AP-1, E4F, ATF-2, and E1A. Additionally, splicing changes induced by an alteration in the activity of a splicing regulator is likely, as E4orf4/PP2A interactions alter the splicing pattern of late viral mRNAs through the dephosphorylation of SR proteins, which are splicing regulators. Bcl-2, a core apoptosis inhibitor, is both positively and negatively regulated by phosphorylation. Similarly, the Bad death-promoter is inactivated by phosphorylation, while two kinases involved in inactivating Bad (PDK1 and AKT) are activated via phosphorylation. Hence, an increase in Bad dephosphorylation or a decrease in PDK1 or AKT dephosphorylation may lead to cell death. Finally, alteration in the MAP kinase growth signaling pathway is suppressed by E4orf4/PP2A complexes. This is likely to lead to a decrease in growth and survival signals.

Therapeutic uses

Molecules that mimic E4orf4 biological activity or modulate or mimic PP2A biological activity are considered useful in the invention; such molecules may be used, for example, as a therapeutic in the treatment of neoplasms or other cell proliferative diseases.

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To add an E4orf4 or PP2A polypeptide to cells in order to modulate cell proliferation or apoptosis, it is preferable to obtain pure E4orf4 or PP2A protein from cultured cell systems that can express the protein. Delivery of the protein to the affected tissue can then be accomplished using appropriate

5   packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to act as E4orf4 or PP2A agonists and in this manner produce a desired physiological effect. Methods for finding such molecules are provided herein.

Gene therapy is another potential therapeutic approach in which nucleic

10 acid encoding E4orf4 or PP2A sense RNA is introduced into cells. The gene must be delivered to those cells in a form in which it can be taken up and encode for sufficient protein to provide effective function.

Transducing retroviral, adenoviral, and human immunodeficiency viral (HIV) vectors can be used for somatic cell gene therapy especially because of

15 their high efficiency of infection and stable integration and expression (see, for example, Cayouette and Gravel, Hum. Gene Ther., 8:423-430, 1997; Kido et al. Curr. Eye Res., 15:833-844, 1996; Bloomer et al., J. Virol., 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; Miyoshi et al., Proc. Natl. Acad. Sci. USA, 94:10319-10323, 1997). For example, the full length E4orf4

20 nucleic acid, or portions thereof, can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as neurons). Other viral vectors which can be used include adenovirus, adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such

25 as Epstein-Barr Virus.

Gene transfer could also be achieved using non-viral means requiring infection in vitro. This would include calcium phosphate, DEAE dextran,

electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are of lower efficiency.

5   Transplantation of normal genes into the affected cells of a patient can also be useful therapy. In this procedure, an E4orf4 nucleic acid sequence is transferred into a cultivatable cell type, either exogenously or endogenously to the patient. These cells are then injected into the targeted tissue(s).

In the constructs described, expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or
10   metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in tumor cells may be used to direct E4orf4 expression. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression.

15   Another therapeutic approach within the invention involves administration of a recombinant E4orf4 polypeptide (e.g, the one described herein), either directly to the site of a potential or actual cell proliferation event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of E4orf4 or
20   analog depends on a number of factors, including the size and health of the individual patient, but, generally, between 0.1 mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically acceptable formulation.

Administration of compounds having E4orf4 or PP2A biological activity

25   An E4orf4 or PP2A protein, gene, or mimetic, or a modulator of PP2A biological activity may be administered within a pharmaceutically-acceptable

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diluent, carrier, or excipient, in unit dosage form. Conventional

pharmaceutical practice may be employed to provide suitable formulations or

compositions to administer activating PP2A antibodies or compounds that

mimic E4orf4 biological activity or modulate or mimic PP2A biological

5   activity to patients suffering from a cell proliferation disease. Administration

may begin before the patient is symptomatic. Methods well known in the art

for making formulations are found, for example, in Remington: The Science

and Practice of Pharmacy (supra). Formulations for parenteral administration

may, for example, contain excipients, sterile water, or saline, polyalkylene

10   glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated

napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide

copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to

control the release of the compounds. Other potentially useful parenteral

delivery systems for compounds include ethylene-vinyl acetate copolymer

15   particles, osmotic pumps, implantable infusion systems, and liposomes.

Formulations for inhalation may contain excipients, for example, lactose, or

may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl

ether, glycocholate and deoxycholate, or may be oily solutions for

administration in the form of nasal drops, or as a gel.

20   The following examples are to illustrate the invention. They are not

meant to limit the invention in any way.

Example 1: Generation of mutant E4orf4 proteins

        To define those areas of E4orf4 required for binding PP2A, and to

define whether the E4orf4-PP2A interaction is required for killing cells, we

25   used a mutagenic approach. We first aligned the amino and sequence of a

number of adenovirus serotypes to determine which amino acids were

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conserved. As shown in Fig. 1, a large number of amino acid residues within the E4orf4 protein are conserved among four different serotypes of adenovirus. We then used PCR based site-directed mutagenesis, according to standard methods known in the art, to generate mutations in the E4orf4 protein of

5 adenovirus serotype Ad2. The complete genome of Ad2 adenovirus is found at GenBank locus ADRCG (NID No. g209811). The Ad2 E4orf4 protein was tagged with a hemagglutinin (HA) tag, such that the expressed protein could be recognized with a commercially available anti-HA tag antibody.

We made a series of point mutants with alanine substitutions at the

10 conserved residues. Since E4orf4 is a small protein of only 114 amino acids, we were able to mutate all of the conserved residues to alanine, each of which is indicted in Fig. 2. At the bottom of Fig. 2 (below the E4orf4 schematic) is a series of single point mutations that we generated. We also generated some grouping of point mutants, which are shown on the top of Fig. 2. For example,

15 we generated a mutant E4orf4 protein in which each of the proline residues at positions 7, 9, and 10 were replaced with alanine residues.

To verify that all of our mutants were stable mutations, we transfected (by the lipofection technique) the human p53-mutant cervical carcinoma line, C33A (commercially available form the American Type Culture Collection

20 (Accession #HTB-31) (ATCC; Manassas, VA). As shown in Fig. 3, we found all of our point mutants to be stable and well-expressed in transfected C33A cells, with the exception of a triple proline mutant at the N-terminus, which is proline rich. All of the single proline point mutants were stable and well-expressed in transfected C33A cells.

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Example 2: Functional analysis of E4orf4 mutants

E4orf4 is known to associate with the B subunit of PP2A (PP2A). While there are a number of B subunits of PP2A, in three separate families, E4orf4 only seems to interact with the Bα subunit. A schematic representation

5   of the interaction of E4orf4 with PP2A is shown in Fig. 4A.

For the studies described below, we used both the human C33A cervical carcinoma line and H1299 cells, a human p53-minus lung carcinoma line (also available from the ATCC) (Accession #CRL-5803). These cells were transfected by lipofection with a FLAG-tagged Bα construct and one of the

10   HA-tagged E4orf4 mutant protein-encoding constructs generated as described above. Since the constructs carried the neo gene (which confers resistance to G418 in transfected cells), we performed G418 drug selection-based colony assays to assess the growth suppressive effect of each mutant E4orf4 protein. E4orf4 immunoprecipitations with anti-HA tag antibody (BAbCO, Richmond,

15   CA) were performed, followed by an *in vitro* phosphatase assay of PP2A activity using a commercially available synthetic phospho-peptide containing a generic PP2A consensus site. Phosphate release was determined spectrophotometrically using malacite green. The same samples were then separated by SDS-PAGE, and blotted for the Bα subunit of PP2A with

20   commercially available anti-Flag antibody (Sigma, St. Louis, MO) to assess binding. In parallel, Western blotting analysis using anti-Flag antibody was performed on whole cell extracts to verify expression of the E4orf4 mutants and the PP2A Bα subunit.

Shown in Fig. 4C are the results from the phosphatase assay. The

25   presence of phosphatase activity in the E4orf4 (anti-HA) immunoprecipitations confirmed that the PP2A holoenzyme associated with E4orf4. The majority of the E4orf4 mutants were associated with a phosphatase activity that was

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roughly equivalent to wild-type E4orf4 (HAorf4). Strikingly, there were a number of mutants that were highly defective in immunoprecipitating active phosphatase.

Following the phosphatase assay, the E4orf4 immunoprecipitation samples were resolved by SDS-PAGE and immunoblotted for the presence of Flag-tagged Bα (Fig. 4D). As a control for lipofection efficiency variations, we also looked at Bα expression in whole cell extracts (WCE) from parallel plates of lipofected cells. As can be seen in Fig. 4D, the Bα expression was consistently seen to be roughly equivalent among the WCE samples, indicating that the lipofection efficiency was roughly equal among the different transfected cells. In the immunoprecipitations, most E4orf4 mutants bound the PP2A Bα subunit with an affinity similar to wild-type E4orf4; however, a number of mutants were either partially or completely defective in PP2A Bα binding.

Fig. 5 is a schematic diagram showing the summary of the mutational analysis of E4orf4 protein. The mutations shown in black in Fig. 5 completely disrupted the ability of E4orf4 to bind to Bα. As expected, these mutants also failed to bring phosphatase activity in E4orf4 immunoprecipitates. The mutations shown in diagonally hatch bars had a reduced ability to bind Bα, and showed a corresponding drop in phosphatase activity. In our experiments, binding to Bα correlated exactly with phosphatase activity in our *in vitro* phosphatase assay.

Our killing data from the colony assays confirmed that PP2A binding was necessary for colony suppression. Mutant that lost PP2A binding (black bars, Fig. 5), also completely lost killing ability, as determined by a lack of colony suppression in G418-resistant stably transfected cells. Likewise, all of the mutants that killed (i.e., suppressed colony formation) also retained PP2A

- 40 -

binding activity. Furthermore mutants with intermediate binding levels (diagonally hatched bars, Fig. 5) were intermediate in their colony suppression abilities. This finding provides very strong evidence that the E4orf4/PP2A association is required to suppress growth in cells. In addition, these findings

5   provided the basis for isolating E4orf4 analogs that also bind the PP2A Bα subunit.

A second important finding that came out of the colony assay was the identification of mutants that were partially defective for colony suppression, but wild-type for Bα binding (indicated as horizontally hatched bars, Fig. 5).

10   This finding indicated that there are multiple requirements for the growth suppression: PP2A binding is required, but not sufficient. To determine if the additional residues are involved in activating the enzyme against particular substrates which may not have been revealed in our broad spectrum phosphatase assay, or if they may be involved in recruiting substrates through

15   direct binding or by altering PP2A localization, one may analyze the subcellular localization of the E4orf4 mutants, as well as screen for other E4orf4 binding proteins. In addition, one may look into the activation of PP2A by E4orf4 with more physiological substrates, such as Rb protein, vimentin, casein kinase II, Bcl-2, and SR proteins.

20   Example 3: E4orf4 kills tumor cells specifically

We next looked at the specificity of killing by E4orf4. Since earlier work had indicated that E4orf4 killed well in cooperation with E1A, we analyzed which oncogene-carrying human tumor lines were susceptible to E4orf4 killing, and, importantly, if normal human cells were resistant to

25   E4orf4 killing. We approached this by performing drug resistant colony assays as described above with the E4orf4 mutants.

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As shown on Fig. 6, a number cultured tumor lines were susceptible to killing when stably transfected with E4orf4 (black bars). Note that all of the cell lines in Fig. 6 are commercially available from the ATCC. The E4orf4 susceptible lines were two cervical carcinoma lines (C33A and HeLa), a lung

5   carcinoma (H1299), a bone fibrosarcoma (HT1080), and an osteosarcoma line (Saos-2). All the susceptible lines were p53 mutant or negative, except the HeLa cells which contain p53. In contrast, the normal lung fibroblast line, Mrc-5, was resistant to killing by stable expression of E4orf4. As a negative control for killing in all cell lines, an inactive E4orf4 mutant was used (white

10   bars outlined with black, Fig. 6). In the Mrc-5 cells, Bax, a pro-apoptotic member of the Bcl-2 family that induces cell death in the vast majority of cells, was used as a positive control for killing (stippled bar, Fig. 6).

To allow us to introduce E4orf4 into primary human lines, we next generated an adenovirus vector producing E4orf4 alone, with no other viral

15   genes expressed, using standard cloning procedures known in the art of molecular biology (see, e.g., Ausubel et al., supra). The E4orf4 protein produced by a cell expressing this vector is, thus, substantially purified, since it is not associated with any other adenovirus-encoded proteins. We initially tried to make a constitutively expressed E4orf4 protein, but the E4orf4 toxicity

20   in 293 cells prevented this. To overcome this toxicity, we produced an inducible adenovirus vector system in which the E4orf4 expression level is tightly controllable by titrating the amount of inducer (doxycycline, a tetracycline analogue). Using this system, we found that low levels of E4orf4 were non-toxic in the H1299 lung carcinoma line (0, 1 or 10 ng/mL Dox);

25   moderate levels were slightly toxic (50 or 100 ng/mL Dox); and high levels were much more toxic (250, 500, 1000 ng/mL Dox). This indicates that high levels of E4orf4 protein are critical for good apoptosis induction.

The construction of the inducible adenovirus vector system is outlined in Fig. 7. The first virus (Ad5-TindHAorf4) contains an HA-tagged version of E4orf4 under the control of the tetracycline-inducible promoter. The second virus (Ad5-CMVrtTA) contains the reverse tetracycline transactivator (rtTA), a

5   transcription factor that binds and activates the tetracycline-responsive promoter in Ad5-TindHAorF4 when bound to tetracycline (or doxycycline). The inducible system operates in a wide variety of tumor and normal cells (Fig. 8; also see below).

In H1299 cells, E4orf4 protein expression was monitored by

10   immunoblot in detergent extracts at different doxycycline concentrations (Fig. 9). Cells were harvested 21 hours after infection with the inducible adenovirus vector system expressing HA-tagged E4orf4. E4orf4 expression was monitored over a time course to determine when maximum expression occurred and how long expression lasted. The low expression at the 72 hour

15   time-point is a reflection of the fact that most of the cells are dead at this point.

Fig. 10 shows the results of a growth inhibition assay in which sparsely plated H1299 cells were infected with the inducible E4orf4 virus + the CMV rtTA virus, the CMVrtTA virus only (at double the concentration) as a control for non-specific viral toxicity, an E1B-19K minus virus (a mutant adenovirus

20   which is very toxic in most humans cells due to virus replication in the absence of apoptosis inhibition), or mock infected (no virus used). These samples were then treated with varying concentrations of doxycycline (matching the amounts used for the immunoblot) and live cells were counted daily under the microscope using a haemocytometer. Cells expressing rtTA showed little or

25   no decrease in cell number, while there was a dramatic reduction in the number of cells expressing rtTA and E4orf4 (Fig. 10).

Using the same inducible adenovirus vector system, a large number of human tumor lines were assayed for their sensitivity to killing by E4orf4 (Fig. 11 and Table 1). The same method for scoring cell growth used for Fig. 10 was used for many of the cell lines (indicated by *). The other group of cells

5   showed poor E4orf4 expression with the tetracycline-inducible system, and displayed greater viability, so they were further analyzed by colony assay. For the colony assay, cells were lipofected with a plasmid expressing E4orf4 plus a drug resistance marker for G418. After lipofection, cells were grown in the presence of G418, killing all cells that were not transfected. Surviving cells

10   were then counted after one to two weeks of drug selection. Forced expression of a toxic gene (e.g., that encoding E4orf4) will kill cells during drug selection even though the cells are resistant to the selection drug. This killing will be reflected by the cell count at the end of the process. In tumor cell lines, plasmids expressing E4orf4 routinely exhibit 10-20% of the cells found in

15   comparison to a control plasmid lacking E4orf4.

TABLE 1

Cell line	Derived from	ATCC accession #
H1299*	large cell carcinoma	CRL-5803
U-2 OS*	osteogenic sarcoma	HTB-96
U-373 MG*	glioblastoma	HTB-17
Hep-3B*	hepatocellular carcinoma	HB-8064
BT-549*	mammary carcinoma	HTB-122
T-24*	bladder cancer	HTB-4
C-33A*	cervical carcinoma	HTB-31
HT-3*	cervical carcinoma, metastatis	HTB-32
SiHa*	cervical carcinoma, squamous	HTB-35
CaSki*	cervical carcinoma, epidermoid	CRL-1550
NCI-H292*	lung carcinoma, mucoepidermoid	CRL-1848
NCI-2030*	non small cell lung carcinoma	CRL-5914
HeLa	adenocarcinoma cervix, epithelial	CCL-2
KB	mouth carcinoma, epithelial	CCL-17
HT1080	fibrosarcoma, epithelial	CCL-121
Saos-2	osteogenic sarcoma, epithelial	HTB-85
A-549	lung carcinoma, epithelial	CCL-185
PC3	prostate adenocarcinoma, epithelial	CRL-1445
SW480	colorectal carcinoma	CCL-228
MS-751	cervical carcinoma, epidermoid	HTB-34

Five cell lines were not killed by tet-induced E4orf4 expression: U-87

MG glioblastoma; MDA-MB-231 epithelial breast adenocarcinoma; SK-OV-3

ovarian adenocarcinoma; 5637 epithelial bladder carcinoma; and NCI-H460

lung large cell carcinoma. Expression of E4orf4 in these cell lines was lower

in these cell lines than that seen in H1299 cells. Thus, the inability of E4orf4

to kill these five cell lines may reflect inadequate E4orf4 protein levels. If

these cell lines are resistant to E4orf4, they serve as control cell lines, for

example, for screening for mimetics of E4orf4.

In contrast to the tumor cell lines, none of the normal primary human

cell lines (Clonetics, San Diego, CA) were susceptible to E4orf4-induced

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killing (Fig. 12 and Table 2). Using the inducible adenovirus vector system, normal cells were found to continue growing in the presence of E4orf4 or at worst exhibit slowed growth. In these normal cells, cell killing was not observed.

5

TABLE 2

Name	Cell type
NHLF	lung fibroblasts
NHDF	dermal fibroblasts
HMEC	mammary epithelial cells
PrEC	prostate epithelial cells
HRE	renal epithelial cells
NHBE	bronchial epithelial cells
CoSmC	colon smooth muscle cells
CoEC	colon endothelial cells
NHEK*	epidermal keratinocytes

*low E4orf4 expression

The results presented in Figs. 11 and 12 indicated that E4orf4 showed some specificity in killing tumor lines (both p53 plus and minus) while normal line was quite resistant. From these experiments, it is clear that E4orf4, recombinantly produced or substantially purified, may be used as a therapeutic agent to induce cell death in tumorigenic cells, while not adversely affecting normal cells. Such E4orf4 proteins, mutants or analogs thereof, or nucleic acids encoding these proteins, having E4orf4 cell death-inducing activity, may be administered according to standard methods with a pharmaceutically acceptable carrier, such as physiological saline or in formulations, such as those described in, for example, in Remington: The Science and Practice of Pharmacy, (19th ed.) ed. A.R. Gennaro AR., 1995, Mack Publishing Company, Easton, PA.

Example 4: E4orf4 expression in *S. cerevisiae* phenocopies PP2A B subunit
deletion mutants

HA-tagged E4orf4 cDNA was subcloned into the episomal yeast vector,
pYES2, under the control of the GAL1 promoter. The GAL1 promoter is

5   repressed in the presence of glucose and induced in galactose, thus allowing
differential expression of E4orf4 based on the carbon source contained in the
growth medium.

To determine if E4orf4 expression had any effects on colony growth,
spotting assays were performed comparing empty pYes2 vector, E4orf4,

10   E311.6K, and Bax (Fig. 13A). E311.6K is another adenovirus protein
believed to be involved in viral induced cell death, while Bax is a cellular
pro-apoptotic protein which has previously been shown to be lethal in yeast.
Fig. 13B shows that all proteins were expressed at high levels. It was found
that E4orf4 expression significantly delayed colony growth compared to the

15   vector control. The E311.6K protein had some minor effect on growth. As
demonstrated in previous studies, Bax expression resulted in a dramatic
cessation of colony growth. E4orf4 expressed in the SEY6210 diploid strain
or EGY48 and BY4742 haploid yeast strains also yielded similar slow growth
effects.

20   To assess whether or not E4orf4 was at all lethal to yeast, a trypan blue
viability assay was done as a time course comparing cells expressing E4orf4
and vector alone. Cells were cultured in glucose, transferred to glycerol, and
approximately equal numbers of cells were then resuspended in either
galactose- or glucose-based medium. Cells expressing E4orf4 were almost all

25   viable at all time points tested from 0 to 108 hours post-induction (Figs. 14A
and 14B).

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Furthermore, at 24 hours post-galactose induction, 30-40% of E4orf4-expressing cells displayed an abnormal elongated morphology (compare Figs. 15A and 15B) highly similar to the morphology observed in *cdc55* strains at low temperatures (Fig. 15C). Cdc55 shares 47% identity and
5   61% similarity with the human Bα subunit. This common morphological phenotype indicated that E4orf4 may be targeting Cdc55, the yeast homolog of the mammalian Bα subunit, providing a molecular link between E4orf4's function in mammalian cells and yeast.

We provide evidence herein that the ability of E4orf4 to induce
10   apoptosis in mammalian tumor cell lines correlates with binding to the Bα subunit of PP2A. Due to the unavailability of a Bα knock-out cell line, however, it has not been shown that the presence of Bα is absolutely required for E4orf4 function. To demonstrate such a direct requirement, we have generated a *cdc55* strain that allows replacement of the *CDC55* gene with a
15   kanamycin cassette. Spotting

assays were then performed using the *cdc55* strain transformed with either vector or E4orf4. We found that in the absence of *CDC55*, the E4orf4-induced growth delay was almost completely abolished (Fig. 16A). This indicates that E4orf4 mediates its effects in yeast through the Cdc55 subunit and that the
20   observed phenotype is not simply due to the overexpression of an exogenous protein. In addition, we repeatedly observed that the expression of E4orf4 is much greater in the *cdc55* deletion strain than in wild type cells (as shown in Fig. 16B). This is consistent with the observed E4orf4-induced toxicity in wild-type cells in which Cdc55 is intact, as it is conceivable that these cells
25   would attempt to downregulate E4orf4 expression. In contrast, in the *cdc55* strain, E4orf4 expression is much greater and the toxicity to yeast is dramatically decreased due to the absence of its cellular target.

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The genetic interaction between E4orf4 and Cdc55 shown above was confirmed biochemically with co-immunoprecipitation experiments (Figs. 17A-17F). Cells containing a *CDC55* disruption were co-transformed with HA-tagged E4orf4 and Flag-tagged Cdc55 or the appropriate controls. Whole

5   cell extracts were prepared 24 hours post-galactose induction and incubated with either anti-HA or anti-Flag antibodies. E4orf4 and Cdc55 co-immunoprecipitate, as shown in the immonoblots presented in Figs. 17B and 17D. In the *cdc55* yeast strain co-expressing E4orf4 and Cdc55, we also found that the A subunit, encoded by *TPD3*, could be identified in

10 HA-immunoprecipitates by Western blotting with rabbit polyclonal anti-Tpd3 antisera. The Tpd3 subunit was not found in the *cdc55* strain containing E4orf4 expressed alone (compare lanes 2 and 4 of Fig. 17C), suggesting that E4orf4 absolutely requires the presence of Cdc55 to recruit PP2A.

To further characterize the E4orf4-PP2A complex in yeast, cells

15 containing E4orf4 plasmid were transformed with either Flag-tagged *PPH21* or *PPH22*, which encode the PP2A catalytic subunits in *S. cerevisiae* (Figs. 18A-18I). Immunoprecipitation with HA antibody followed by western blotting with anti-Flag revealed that both Pph21 and Pph22 can be immunoprecipitated with E4orf4 (Fig. 18B). The reciprocal experiment,

20 immunoprecipitating with Flag-antibody and blotting against the HA epitope, was also done to confirm this interaction (Fig. 18D). Reprobing these membranes with anti-Tpd3 showed the A subunit was also present in both HA and Flag precipitates (Figs. 18C and 18F). From the above studies we conclude that, through direct or indirect binding of the Cdc55 subunit, E4orf4

25 can associate with the PP2A holoenzyme.

We were interested to know if E4orf4 alanine point mutants, previously characterized in human cell lines based on their ability to bind Bα, had an

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analogous phenotype in yeast. R81/F84 does not bind Bα and does not kill tumor cells. K88 binds Bα, but has no effect in cells. These and other mutants were tested in wild type haploid yeast for colony growth, morphology, and Cdc55 binding (Figs. 19A-19C). As shown in Fig. 19B, all mutants were

5 stably expressed. The spotting assay results show that S106 has a similar effect on colony growth as did wild-type E4orf4. In contrast, both K88 and R81/F84 had an intermediate effect on growth (Fig. 19A). Cells from the plates were collected and examined for cell morphology. Cells expressing the R81/F84 mutants appeared to have normal

10 yeast morphology, resembling those containing vector alone. Cells expressing the K88 mutant displayed largely normal morphology, although a few cells (<1%) did have an elongated cell morphology.

To determine if E4orf4 point mutants were able to interact with Cdc55, co-immunoprecipitation experiments were performed in the *cdc55* strain

15 transformed with wild-type E4orf4 or the E4orf4 point mutants (S106, K88 and R81/F84) and CDC55 (Fig. 19C). The results demonstrated a greatly reduced interaction between Cdc55 and the K88 mutant and no interaction with the R81/F84 mutant. Although there seems to be some weak interaction between K88 and Cdc55, this mutant behaves essentially as the R81/F84

20 mutant in yeast with respect to colony growth. This difference in the behavior of this E4orf4 mutant is possibly due to sequence-specific differences between mammalian Bα and yeast Cdc55 subunits.

E4orf4 can associate with all four members of the B class regulatory subunits, but interaction with the B' or B" classes have not been detected. The

25 only other B regulatory subunit known to exist in budding yeast is Rts1, which has homology to the B' class. To determine if the B' class subunit of yeast is required for the effect elicited by E4orf4 in yeast, we tested the effects of wild-

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type E4orf4 and E4orf4 mutants in an *rts1* strain (Figs. 20A and 20B).
Haploid wild type yeast or yeast containing an *RTS1* deletion were transformed
with the indicated plasmids and spotting assays were performed (Fig. 20A).
Fig. 20B shows that all proteins were expressed in the deletion strain. While

5   the slow growth effect of wild type E4orf4 was essentially abrogated in the
cdc55 strain, the growth delay was maintained in the *rts1* strain. The E4orf4
mutants also displayed the intermediate growth delay in the *rts1* strain. We
concluded that the Cdc55 subunit (B) is required by E4orf4 to elicit an effect
in yeast while the Rts1 subunit is dispensable for E4orf4 function in yeast.

10   The foregoing data indicate that *S. cerevisiae* can serve as a useful organism
for performing screens to identify E4orf4 analogs or mimetics that modulate
PP2A biological activity.

Other Embodiments

All publications and patents mentioned in the above specification are

15   herein incorporated by reference. Various modifications and variations of the
described method and system of the invention will be apparent to those skilled
in the art without departing from the scope and spirit of the invention.
Although the invention has been described in connection with specific
preferred embodiments, it should be understood that the invention as claimed

20   should not be unduly limited to
such specific embodiments. Indeed, various modifications of the described
modes for carrying out the invention which are obvious to those skilled in
molecular biology or related fields are intended to be within the scope of the
invention.

25

We Claim:

1. A method for identifying a compound as one useful for the treatment of cancer, said method comprising:

(a) providing a first cell that has been modified to have no protein phosphatase 2A biological activity and a second cell that has not been modified to have no protein phosphatase 2A biological activity;

(b) contacting said cells with a candidate compound; and

(c) assaying said cells for cell growth,

wherein a decrease in cell growth of said second cell and no decrease in cell growth of said first cell identifies said candidate compound as a compound useful for the treatment of cancer.

2. A method for identifying a compound as one useful for the treatment of cancer, said method comprising:

(a) providing a first cell that has been modified to have no protein phosphatase 2A biological activity and a second cell that has not been modified to have no protein phosphatase 2A biological activity;

(b) contacting said cells with a candidate compound; and

(c) assaying said cells for cell death,

wherein an increase in cell death of said second cell and no increase in cell death of said first cell identifies said candidate compound as a compound useful for the treatment of cancer.

3. The method of claim 1 or 2, wherein said first cell has a mutation in a gene encoding a subunit of protein phosphatase 2A, said mutation resulting in no protein phosphatase 2A biological activity.

4. The method of claim 3, wherein said subunit is a B subunit.

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5. A method for identifying a compound useful for the treatment of cancer, said method comprising the steps of:

a) providing a cell expressing a protein phosphatase 2A B subunit;

b) contacting said cell with a candidate compound; and

c) measuring binding of said compound to said protein phosphatase 2A B subunit, wherein said binding of said compound to said protein phosphatase 2A B subunit identifies said candidate compound as a compound that is useful for the treatment of cancer.

6. A method for identifying a compound useful for the treatment of cancer, said method comprising the steps of:

a) providing a cell expressing a protein phosphatase 2A B subunit;

b) contacting said cell with a candidate compound; and

c) measuring biological activity of protein phosphatase 2A in said cell, wherein a change in said biological activity relative to a cell not contacted with said candidate compound identifies said candidate compound as a compound that is useful for the treatment of cancer.

7. The method of claim 1, 2, 5, or 6, wherein said cell is a mammalian cell.

8. The method of claim 1, 2, 5, or 6, wherein said cell is a yeast cell.

9. The method of claim 8, wherein said yeast is *Saccharomyces cerevisiae.*

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10. A method for identifying a compound useful for the treatment of cancer, said method comprising the steps of:

(a) contacting a candidate compound with a protein phosphatase 2A B subunit; and

(b) measuring binding of said compound to said protein phosphatase 2A B subunit, wherein a change in said binding identifies said candidate compound as a compound that is useful for the treatment of cancer.

11. A method for identifying a compound useful for the treatment of cancer, said method comprising the steps of:

(a) contacting a candidate compound with a protein phosphatase 2A B subunit; and

(b) measuring biological activity of protein phosphatase 2A in said cell, wherein a change in said biological activity relative to a cell not contacted with said candidate compound identifies said candidate compound as a compound that is useful for the treatment of cancer.

12. A method of increasing cell death in a cell, said method comprising administering a compound that modulates protein phosphatase 2A dephosphorylation of a protein phosphatase 2A substrate.

13. The method of claim 12, wherein said protein phosphatase 2A substrate is selected from the group consisting of Rb protein, vimentin, casein kinase II, Bcl-2, or an SR protein.

14. A method of increasing apoptosis in a cell, said method comprising administering to said cell an apoptosis-inducing amount of a protein phosphatase 2A subunit or cell death-inducing fragment thereof.

15. The method of claim 12 or 14, wherein said cell is in a mammal.

16. The method of claim 15, wherein said mammal is a human.

17. The method of claim 16, wherein said human has a disease involving excessive cell growth.

18. The method of claim 17, wherein said disease is cancer.

19. A pharmaceutical composition comprising (i) a substantially pure mammalian protein phosphatase 2A subunit or a cell death-inducing fragment thereof; and (ii) a pharmaceutically acceptable carrier.

20. A method for identifying a compound useful for the treatment of cancer, said method comprising the steps of:
    a) providing a cell expressing protein phosphatase 2A;
    b) contacting said cell with a candidate compound; and
    c) measuring binding of said compound to protein phosphatase 2A, wherein said binding of said compound to said protein phosphatase 2A identifies said candidate compound as a compound that is useful for the treatment of cancer.

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                    10         20         30         40         50

SCORE         1   ▨  ▨▨  ▨      ▨    ▨  ▨▨▨ ▨▨ ▨  ▨▨ ▨ ▨▨   ▨ ▨ ▨▨▨▨
CONSENSUS     1   MxLPxLPPPP VSxLPxCIx  WLGLAYxxCV DVxRxIxHxG VxISPEAEEL
Ad5 E4orf4    1   MVLPALPAPP VCDSQNECVG WLGVAYSAVV DVIRAAAHEG VYIEPEARGR
Ad9 E4orf4    1   MVLPILPPPP LNDRQGSIN- WMGMAYRVLA DVMRGIRMDG FFISSDAEEL
Ad12 E4orf4   1   MPLPCIPPPP VSRDTAACIA WLGLAHASCV DTLRFIKHHD LKITPEAEYI
Ad40 E4orf4   1   MPLPSLPPPP VSRDQGLCIS WLELALTCCL NVYGDIIRYN VSISPRAEEL

SCORE        51   ▨  ▨▨    ▨  ▨   ▨ ▨ ▨       ▨     ▨ ▨▨ ▨ ▨▨    ▨
CONSENSUS    51   LxxLREWLYF AxxTERQRRx DRRRxRICxx RTWFCFQKYx xVRKSIxYDA
Ad5 E4orf4   51   LDALREWIYY NYYTERSKRR DRRRRSVCHA RTWFCFRKYD YVRRSIWHDT
Ad9 E4orf4   51   LQNLREWMYF SWMTERQQRK DGRRRGICCS RAHFCWQKYD KVRKRIHYNA
Ad12 E4orf4  50   LASLREWLYF AFLTERQRCK QKGRGAITSG RTWFCFFKYE DARKSVVYDA
Ad40 E4orf4  51   LSGLQEWLNV ALKTERARQR NRRFANICWQ RVRFLKQKYE AVRELLIYDA

SCORE       101   ▨         ▨   ▨ ▨  ▨ ▨ ▨     ▨
CONSENSUS   101   TRQTxSLQxx xSxQxPxxTx L
Ad5 E4orf4  101   TTNTISVVSA HSVQ----- -
Ad9 E4orf4  100   NRDSIQLAPP SSISQGPFTT I
Ad12 E4orf4 101   ARQTVSLQIG TIQQVPT-TA L
Ad40 E4orf4 101   TRQMVSLQTE FVPNPPSSTS L
```

Fig. 1

Fig. 2

Fig. 3

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4D

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Fig. 5

Fig. 6

Fig. 7

Fig. 8
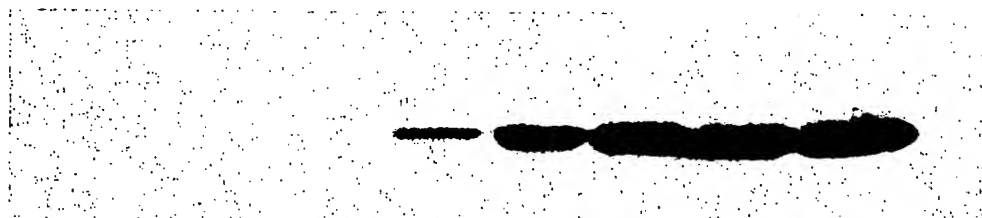
Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13A

Fig. 13B

Fig. 14A

Fig. 14B

Fig. 15A

Fig. 15B

Fig. 15C

Fig. 16A

Fig. 16B
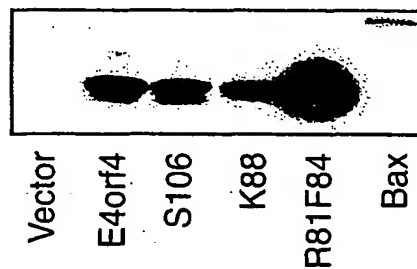
Fig. 17

Fig. 18

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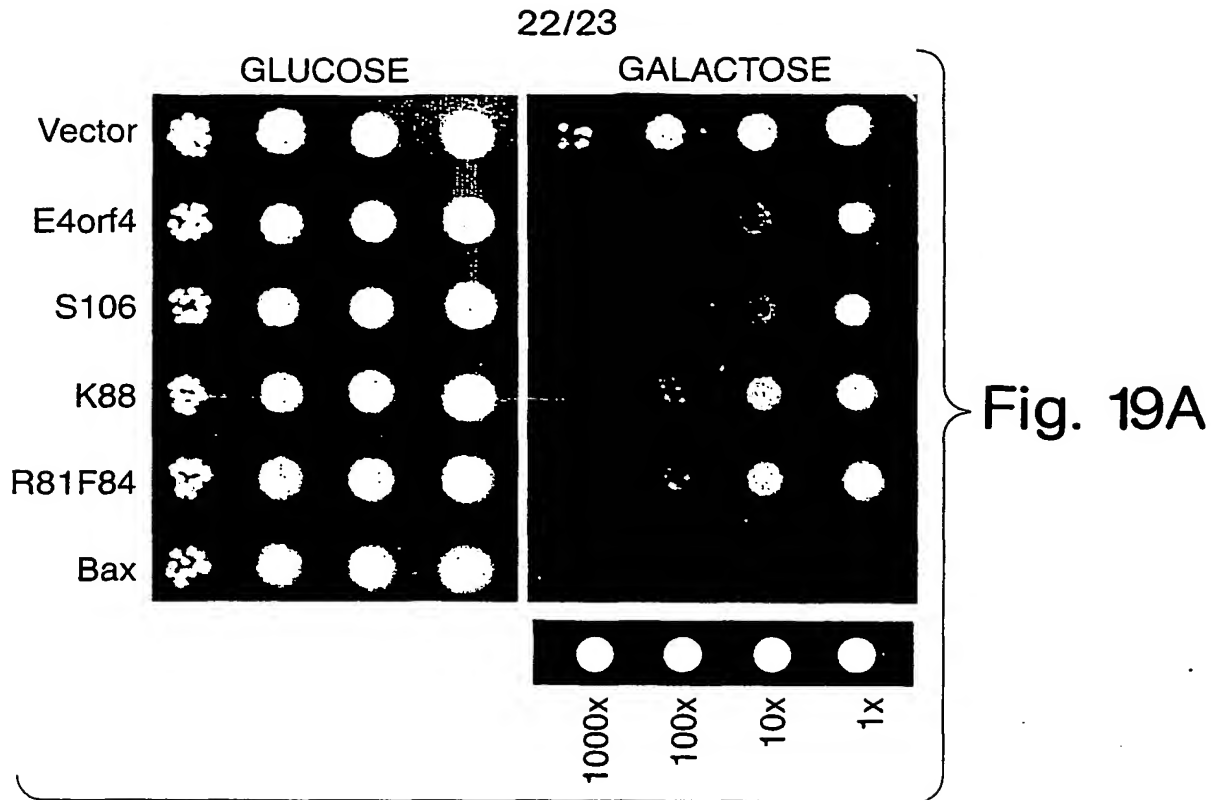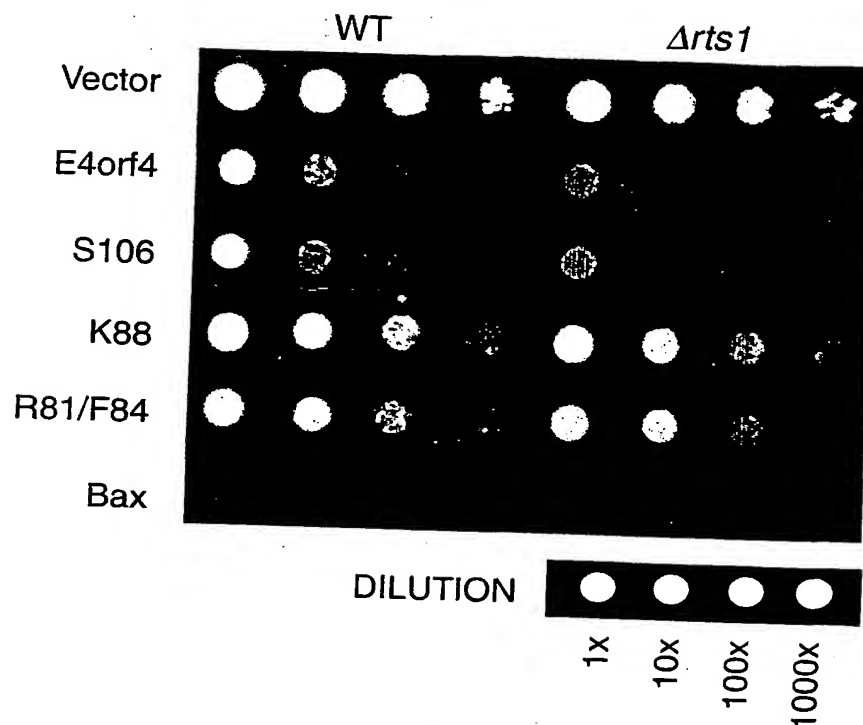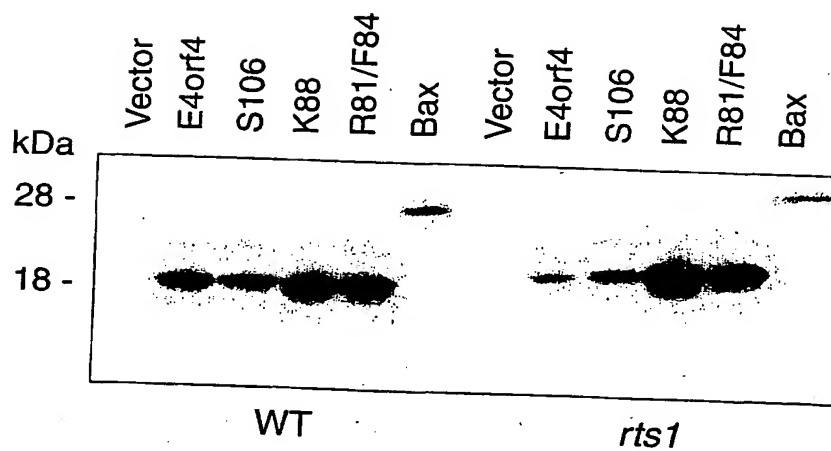Fig. 19A

Fig. 19B

Fig. 19C

Fig. 20A

Fig. 20B

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00817

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   G01N33/50   C12Q1/02   C12Q1/42   A61K38/46   A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   G01N   C12Q   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 35124 A (THOMAS JEFFERSON UNIVERSITY) 7 November 1996 (1996-11-07)	1-11,20
X	the whole document	12,13, 15-18
A	EP 0 874 052 A (BIOPHARM GESELLSCHAFT ZUR BIOTECHNOLOGISCHEN ENTWICKLUNG VON PHARMAKA) 28 October 1998 (1998-10-28)	1-11,20
X	the whole document	14,19
A	WO 98 01563 A (P. E. BRANTON ET AL.) 15 January 1998 (1998-01-15) cited in the application page 34, line 23 -page 35, line 10 page 40, line 9 -page 41, line 25 page 45, line 25 -page 46, line 12	1-11,20
X	claim 1	12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2000	01/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. WIPF ET AL.: "Combinatorial synthesis and biological evaluation of library of small-molecule Ser/Thr-protein phosphatase inhibitors" BIOORGANIC & MEDICINAL CHEMISTRY., vol. 5, no. 1, 1997, pages 165-177, XP002119507 ELSEVIER SCIENCE LTD., GB ISSN: 0968-0896 the whole document	1-20
A	WO 97 37037 A (NOVARTIS AG) 9 October 1997 (1997-10-09) the whole document	1-20
T	R. SHTRICHMAN ET AL.: "Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 96, no. 18, 31 August 1999 (1999-08-31), pages 10080-10085, XP002153092 WASHINGTON US the whole document	1-20

INTERNATIONAL SEARCH REPORT

information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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WO 9801563	A	15-01-1998	AU CA EP	3860197 A 2259152 A 0951553 A	02-02-1998 15-01-1998 27-10-1999
WO 9737037	A	09-10-1997	AU EP	2027997 A 0889971 A	22-10-1997 13-01-1999

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